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Age-Specific Prevalence and Genetic Diversity of GBV-C/Hepatitis G Virus in Brazil

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The recently discovered GBV-C/hepatitis G virus (GBV-C/HGV) has been shown to be parenterally transmitted. The occurrence of community-acquired GBV-C/HGV infections has also been reported. In order to study the variations with age of the GBV-C/HGV prevalence, sera from 268 individuals without liver disease, aged 0–80 years, and living in the city of Rio de Janeiro, Brazil, were tested by reverse transcription-nested polymerase chain reaction for the presence of GBV-C/HGV RNA. Age-specific seroprevalence was low (2.3%) among children under the age of 10, reached a maximum of 18% in young adults (21–30 years), and declined in older age groups. Among 170 blood donors aged 18–60, the rate of individuals with antibodies against the viral envelope E2 protein increased with age, from about 6% between the ages of 18 and 24 to about 35% for individuals from the age of 43 to 60. The results suggest that sexual transmission of GBV-C/HGV might occur and that the virus could be eliminated after a long period of infection. The nucleotide sequences of GBV-C/HGV genome fragments, 422 base pairs (bp) in the E2 region and 354 bp in the nonstructural 5 region, were determined for 11 and 31 isolates, respectively. Phylogenetic tree based on concatenated E2+NS5 sequences showed that Brazilian strains belonged to three clusters, two of which were previously characterized as genotypes 1 and 2. ***J. Med. Virol. 56: 39–43, 1998.***

KEY WORDS: GVB-C; HGV; age distribution; nonstructural 5 region; E2 region; phylogenetic analysis

INTRODUCTION

Following the discovery of hepatitis C virus (HCV), which is responsible for most of the non-A, non-B hepatitis cases, a new human flavivirus, named GB virus C (GBV-C) or hepatitis G virus (HGV), was identified from the serum of patients with non-A–C hepatitis [Simons et al., 1995; Linnen et al., 1996]. The virus has a worldwide distribution. Although a possible role of GBV-C/HGV in the etiology of fulminant hepatitis has been suggested, the virus has not been definitively associated with any disease. Epidemiological studies have shown that GBV-C/HGV can be transmitted parenterally. Mother-to-infant [Feucht et al., 1996; Moaven et al., 1996; Viazov et al., 1997b] and sexual [Kao et al., 1997] routes of transmission have also been described. However, the relative importance of each route of transmission has not yet been determined.

GBV-C/HGV RNA contains approximately 9,400 nucleotides. The relative positions of the structural and nonstructural genes in the GBV-C/HGV genome are similar to those of HCV. However, the GBV-C/HGV genome lacks a clearly identifiable core gene [Muerhoff et al., 1996] and hypervariable regions within the envelope coding sequences [Erker et al., 1996]. The genomes of at least 17 independent isolates from Africa, Asia, and North America have been fully sequenced, showing a similarity higher than 85%, far exceeding that reported for HCV genomes. GBV-C/HGV thus appears to be significantly less variable than HCV [Viazov et al., 1997a]. Its mutation rate of 3.9×10^{-4} base substitutions per site per year is fourfold below that of HCV [Nakao et al., 1997].

The distribution of GBV-C/HGV samples from around the world into three or more groups (or genotypes) has been suggested recently [Fukushi et al., 1996; Muerhoff et al., 1996; González-Perez et al., 1997; Katayama et al., 1997; Mukaide et al., 1997; Okamoto et al., 1997; Smith et al., 1997] and a correlation between genotype and geographical origin of the variants has been observed. Partial nucleotide se-

The sequences reported in this paper have been deposited in the Genbank database under accession numbers AF060823 to AF060833 (E2 sequences) and AF061659 to AF061683 (NS5 sequences).

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quence has been determined from many samples from North America, Europe, Asia, and Africa, and genotypes 1, 2, and 3 would prevail in Africa, North America, and Japan, respectively. However, little is known about the genetic diversity of GBV-C/HGV strains circulating in South America.

The age distribution of GBV-C/HGV infection in normal individuals (without liver disease) living in Rio de Janeiro, Brazil, and a genotype attribution of identified GBV-C/HGV isolates are described.

MATERIALS AND METHODS

Population Studied

Age-specific seroprevalences of GBV-C/HGV RNA were measured in two populations of individuals living in Rio de Janeiro, Brazil. The first group was composed of 268 individuals aged 0–80 years (mean ± SD = 32 ± 20 years) and without liver disease, including 132 voluntary blood donors and 136 individuals who were referred to an outpatient clinic (including 43 adults with diabetes and 35 children under the age of 10 with exanthematous disease). The second group consisted of a cohort of 113 consecutive patients (mean ± SD = age 52 ± 14 years) with chronic liver disease, 52 (46%) of them being alcohol abusers and 48 (42%) had received blood transfusion. This cohort has been described elsewhere [Lampe et al., 1998]. In addition, a total of 170 sera from voluntary blood donors (the 132 mentioned above plus 38), aged 18–60 years, were used to determine the age-specific seroprevalence of antibodies to the second-envelope glycoprotein (anti-E2).

The partially sequenced GBV-C/HGV genomes originated from patients with chronic liver disease (24 strains) and under maintenance hemodialysis (7 strains). Demographic, clinical, and serological data of patients with chronic liver disease [Lampe et al., 1998] and under hemodialysis treatment [Lampe et al., 1997] have been reported previously.

Detection of Anti-E2 Antibodies

An enzyme immunoabsorbent test, microplate anti-HGenv kit (Boehringer Mannheim, Germany), was used for the determination of anti-E2 antibodies in serum.

RNA Extraction and Polymerase Chain Reaction Amplification

Viral RNA was extracted from serum by using the SNAP total isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions with the following modifications: 10 μg yeast tRNA were added to 150 μl serum, and the final step (removal of DNA) was omitted. One-fifth of the RNA was reverse-transcribed in the presence of Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Life Technologies, Gaithersburg, MD). One-third of the resultant cDNA was used as a template for polymerase chain reaction (PCR) amplification. Nested PCR in the nonstructural (NS) 5 region was carried out using outer primers YK-874 and YK-877, along with second-round primers YK-876 and YK-1183 [Khudyakov et al., 1997]. Sequences of the E2 region were detected after nested PCR using external primers SV8S (5′-CAGYTGGCCTCCAAYTACTGGAT-3′, nt 971–993 on the GBV-C prototype genome) and SV7A (5′-CAGCAGTCCCKCACACAGGTGCC-3′, nt 1,647–1,625), and internal primers SV4S (5′-GTGACNAT-GGCKGGCATGTC-3′, nt 1,118–1,137) and SV5A (5′-GCAVGGGAYYCCCCACTCGGAGAGC-3′, nt 1,588–1,564). PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. Positive samples gave only one band of the expected size (402 bp for NS5 region and 471 bp for E2 region). GBV-C/HGV identity of PCR products was confirmed by nucleotide sequencing.

Cloning and Nucleotide Sequencing

PCR products from NS5 region were cloned into pCRII vector (TA cloning kit, Invitrogen) and the recombinant plasmids were purified by ultracentrifugation in CsCl gradient. Both strands of the insert DNA were sequenced using Cy5-labeled forward and reverse M13 primers and the reagents provided in the Autoread Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden). Sequencing products were analyzed on an ALFexpress automated sequencer (Pharmacia, Piscataway, NJ). PCR products from E2 region were subjected to direct sequencing from both directions using a Prizm Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer, Norwalk, CT).

Computer-Assisted Sequence Analysis

Nucleotide sequences were aligned using PILEUP (Wisconsin Sequence Analysis Package, GCG, Madison, WI). This program uses the unweighted pair-group method with arithmetic averages (UPGMA) procedure. For the construction of dendrograms, our sequences were compared with 17 GBV-C/HGV full-size genome sequences available in databanks. These were from the following isolates: BG1HC, CG01BD, CG07BD, CG12LC, G05BD, and G13HC (Genbank AB003288 to AB003293) [Takahashi et al., 1997]; GBVC-East Africa (U63715) [Erker et al., 1996]; GBVC-West Africa (U36380) [Leary et al., 1996]; GSI85 (D87262) [Nakao et al., 1997]; GT110 (D90600) and GT230 (D90601) [Okamoto et al., 1997]; HGVC964 (U75356); HGVGD (AF006500); HGV-IM71 (AB008342); HGV-Iw (D87255) [Shao et al., 1996]; PNF2161 (U44402) and R10291 (U45966) [Linnen et al., 1996].

Statistical Analysis

The 95% confidence intervals of proportions were calculated using GraphPad Instat statistical program (GraphPad Software, San Diego, CA).

RESULTS

Age-Specific Prevalence of GBV-C/HGV Infection

Twenty-three of 268 sera (8%) from individuals without liver disease were GBV-C/HGV RNA-positive. This

TABLE I. Age-Specific Prevalence of Serum GBV-C/HGV RNA in Individuals Without Liver Disease

Age (years)	n	Prevalence (CI[a]), %
<11	43	2.3 (0.4–12.1)
11–20	33	9.1 (3.2–23.6)
21–30	56	17.9 (10.1–29.9)
31–40	46	13.0 (6.1–25.6)
41–50	40	7.5 (2.6–19.9)
>50	50	0.0 (0.0–7.1)

[a]CI: 95% confidence interval.

TABLE II. Seroprevalence of Anti-E2 Antibodies in Blood Donors According to Age

Age (years)	n	Prevalence (CI), %
18–24	36	5.6 (1.5–18.1)
25–29	34	11.8 (4.7–26.6)
30–35	36	11.1 (4.4–25.3}
36–42	30	30.0 (16.7–45.3)
43–60	34	35.3 (21.5–52.2)

TABLE III. Seroprevalence of GBV-C/HGV RNA in Patients With Chronic Liver Disease According to Age

Age (years)	n	Prevalence (CI) %
<41	23	17.4 (7.0–37.1)
41–50	33	12.1 (4.8–27.3)
51–60	27	11.1 (3.9–28.1)
>60	30	13.3 (5.3–29.7)

was determined by reverse transcriptase (RT)-nested PCR using primers from the NS5 region. Table I shows the age distribution of GBV-C/HGV carriers. The curve of GBV-C/HGV RNA seroprevalence presented a peak at an age range of 21–30 years; 18% of the individuals of this age group were infected. Only one child under the age of 10 (an eight-year-old boy) of 43 tested (2.3%) was GBV-C/HGV RNA- positive. All other GBV-C/HGV carriers were between 15 and 44 years old. No serum sample from adults older than 50 (among 50 sera tested) was found to contain GBV-C/HGV RNA.

One hundred and seventy sera from blood donors aged 18–60 years were tested for the presence of antibodies to the viral envelope E2 protein. In the age group 18–24 years, only 5.6% presented anti-E2 antibodies (Table II). This value increased rapidly with age to reach 30.0% for the population aged 36–42 years and 35.3% for blood donors 43–60 years old. Among 132 sera that were tested for both GBV-C/HGV RNA and anti-E2 antibodies, only two (1.5%) were positive for both markers.

In a population of 113 patients with chronic liver disease, the proportion of GBV-C/HGV RNA-positive patients did not vary significantly with age (Table III), remaining relatively stable from the group of patients under 41 (17.4%) to those older than 60 (13.3%). Markedly, 7 of 57 (12%) patients older than 50 were GBV-C/HGV-infected. However, it should be noted that three had received at least one blood transfusion, which is a possible cause of infection. Three other GBV-C/HGV-infected patients older than 50 years were alcohol abusers.

Genetic Diversity of GBV-C/HGV Isolates

The nucleotide sequences of a 354 bp fragment of the NS5 region of the GBV-C/HGV genome were determined for 31 isolates. These sequences were compared by phylogenetic analysis with GBV-C/HGV sequences (available in databanks) of isolates from around the world. It appeared that three Brazilian strains belonged to the genotype numbered 1 [Muerhoff et al., 1996] or G1 [Okamoto et al., 1997], containing the GBVC-West Africa prototype [Leary et al., 1996]. However, genotypes 2 and 3 could not be distinguished.

In an attempt to better characterize the GBV-C/HGV genotypes circulating in Brazil, we sequenced a fragment of 422 bp from E2 region. This was carried out with 11 strains. E2 and NS5 sequences were then concatenated and a new phylogenetic analysis was performed. The results appear in Figure 1. Four groups of strains (genotypes?) were observed. Two (S019 and S095) and three (S008, S066, and B083) Brazilian strains were classified into groups 1 and 2, respectively. Group 3 was exclusively composed of Asian strains. A fourth cluster was constituted by six of our strains (S031, S054, C043, S4956, A001, and S794) and a GBV-C East-African isolate [Erker et al., 1996]. However, bootstrap resampling (100 replicates) did not confirm the branching order of the phylogenetic tree; the resulting bootstrap values were lower than the generally recognized cutoff value of 75%.

DISCUSSION

The age-specific GBV-C/HGV RNA seroprevalence was evaluated in 268 individuals without liver disease living in Rio de Janeiro. This was carried out by RT-nested PCR using a set of primers designed from the NS5 region and highly efficient for this purpose [Khudyakov et al., 1997]. Except for one eight-year-old child, the youngest individual infected was 15 years old and the curve of prevalence showed a peak at 18% in young adults aged 21–30 years (Table I). These data may suggest a role for sexual transmission of GBV-C/HGV. In a study with healthy individuals aged 16–67 years in Uzbekistan, it has also been shown that GBV-C/HGV infection was distributed among younger people [Kato et al., 1997]. The high value (18%) encountered in age group of 21–30 years was likely due to the persistence of GBV-C/HGV infection, commonly for 5–10 years, as previously reported [Lefrère et al., 1997]. Indeed, such a long period of infection increases the probability of viral RNA detection in a cross-sectional study.

Some reports have suggested anti-E2 seroconversion to be associated with viral clearance [Dille et al., 1997; Tacke et al., 1997]. Our data showing a lower GBV-C/HGV RNA seroprevalence in healthy individuals older than 30 years, in association with a continuing increase with age of the rate of anti-E2 antibodies-positive individuals among blood donors (Table II), are in accordance with that finding. Furthermore, only 2 of

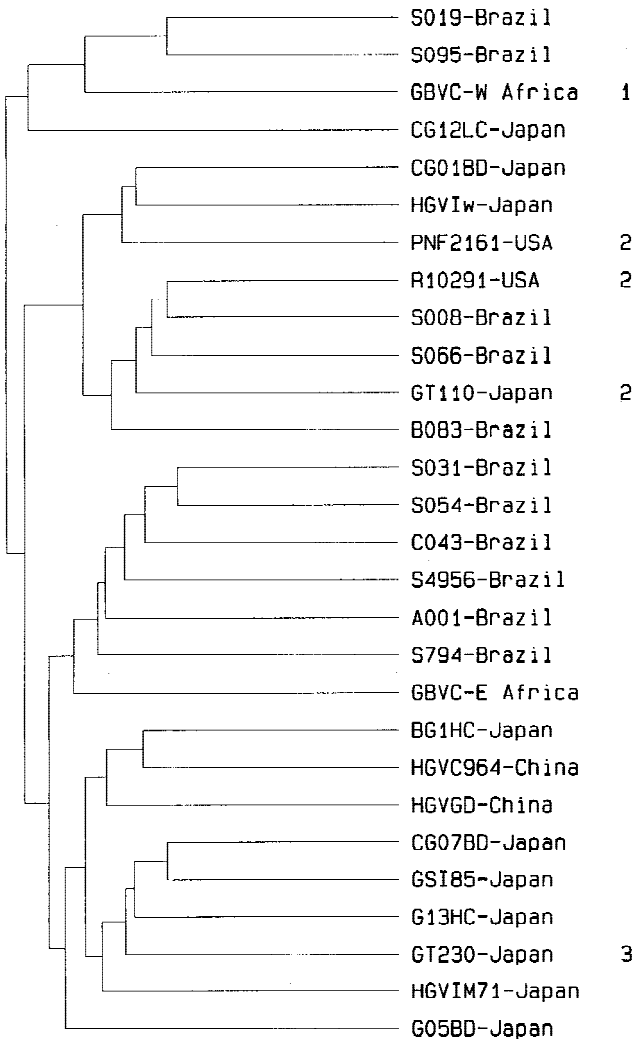

Fig. 1. Phylogenetic tree (UPGMA) of 11 Brazilian and 17 other GBV-C/HGV isolates obtained with concatenated E2 and NS5 sequences. Sequences from these separate regions of the viral genome were linked together to form colinear sequences for each isolate. Numbers at the right correspond to the genotypes of prototype strains as previously established [Okamoto et al., 1997].

132 blood donors were positive for both GBV-C/HGV RNA and anti-E2 antibodies markers.

In the group of patients with chronic liver disease, the mean age (52 years) was higher than in individuals without liver disease. However, the sharp decrease of GBV-C/HGV RNA seroprevalence with increasing age, particularly above 40, that occurred in the group of individuals without liver disease was not observed. In contrast, no marked decrease of the proportion of GBV-C/HGV carriers was observed in patients older than 40 (Table III). This may be due to risk factors to which this particular population has been exposed, such as blood transfusion, and perhaps abuse of alcohol intake. Indeed, an association between GBV-C/HGV infection and excessive alcohol consumption has recently been reported in South Africa [Tucker et al., 1997]. In the present study, of a total of seven patients older than 50 who were infected, three had received blood transfusion and three others were alcohol abusers.

Although GBV-C/HGV is not characterized by a genome variability as high as that of HCV [Nakao et al., 1997], several studies have suggested the existence of at least three distinct genotypes [Fukushi et al., 1996; Muerhoff et al., 1996; González-Perez et al., 1997; Katayama et al., 1997; Mukaide et al., 1997; Smith et al., 1997]. In a recent report, three GBV-C/HGV genotypes have been defined, based on the divergence of >12% in the entire genomic sequence [Okamoto et al., 1997]. Genotypes 1, 2, and 3 were represented by prototype strains GBVC-West Africa, PNF2161-USA, and GT230-Japan, respectively. Strains from genotype 1 would be predominant in West Africa, while genotype 2 has been found in the United States and Europe, and genotype 3 viruses have been isolated in Asia. On dendrogram of Figure 1, the prototype strains of the three genotypes were separated into three different groups. However, it has been found that the distribution of the pairwise evolutionary distances of a large number of GBV-C/HGV partial sequences exhibited a single peak [Viazov et al., 1997a]. This was in contrast to HCV, for which three levels of nonoverlapping distributions were observed, representing types, subtypes, and isolates. The existence of a single genotype of GBV-C/HGV has thus been proposed [Wang et al., 1997]. We carried out a phylogenetic analysis with 17 sequences available in databanks, based on more than half of the genome (nucleotides 1 to 5,000). No clear division in genotypes was obtained (not shown).

A recent report has described GBV-C/HGV strains from Argentina as closely related to North American isolates [Muerhoff et al., 1997]. We found several Brazilian isolates related to North American and European strains (type 2), strains clustering with GBV-C-West Africa (type 1), and some viruses classified into a separate group and genetically related to GBVC-East Africa, which is a strain under classification [Muerhoff et al., 1997]. The presence of strains belonging to 3 of 4 distinct clusters reveals a large genetic diversity of GBV-C/HGVs circulating in Brazil.

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